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12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

The goal of the proposed work is to test the hypothesis that myoepithelial cells play an important role in the regulation of mammary development and differentiation, and are, therefore, a determinant of susceptibility to carcinogenesis. Our preliminary data indicated that K5 E2F-1 transgenic mice had disrupted mammary development, and we proposed to further characterize the mammary phenotype, with the goal of developing a new and beneficial model for mammary cancer research. We proposed that our unique model would be useful in understanding the effects of myoepithelial cells on mammary development because the K5 promoter directs transgene expression in mammary glands exclusively to these basal cells. The overall focus of our work in this model has been: 1) to develop a stable animal model, on a established background, in which to conduct tumorigenesis research, 2) to characterize the stable phenotype(s) and, 3) to conduct the proposed carcinogenesis experiments. Over the past year we have made considerable progress in specific aims 1 & 2; understanding the impact of disruption of the myoepithelial cells produced by directed transgenic overexpression of the cell cycle regulator E2F-1. We conclude at this time that disruption of the mammary myoepithelial cells by E2F-1 transgene overexpression impairs the mammary ductal arborization in virgin animals, and also inhibits the development of the lactational phenotype in pregnant mice. We have particularly made progress in evaluating the genesis/function of myoepithelial cells in the glands from neonates. These data indicate that the phenotypic changes found in the transgenics result from alterations in an established population of K5-positive basal cells present from the time of birth.

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Breast cancer, mammary development, mammary differentiation, lactation, apoptosis

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Introduction

The goal of the proposed work is to test the hypothesis that myoepithelial cells play an important role in the regulation of mammary development and differentiation. We reported our original finding that K5 E2F-1 transgenic mice had disrupted mammary development, and proposed to further characterize the glands, towards the goal of developing a new and useful model for mammary cancer research. We proposed that our model would be useful in understanding the effects of myoepithelial cells on mammary development because the K5 promoter directs transgene expression in mammary glands exclusively to these basal cells. Published reports show that the level of mammary differentiation is an important aspect of the response to chemical carcinogens, and we proposed that the K5 transgenic mice, with reduced levels of mammary differentiation, would demonstrate increased susceptibility to treatment with DMBA. The overall focus of our work in this model has been: 1) to develop a stable animal model, on a established background, in which to conduct tumorigenesis research, 2) to characterize the stable phenotype(s) and, 3) to conduct the proposed carcinogenesis experiments. Over the past year we have made considerable progress in specific aims 1 & 2; understanding the impact of disruption of the myoepithelial cells produced by directed transgenic overexpression of the cell cycle regulator E2F-1. We have particularly made progress in evaluating the genesis/function of myoepithelial cells in the glands of very young mice. This has given us increased confidence that the phenotypic changes that are found in these mice result from alterations in an established population of K5-positive basal cells present from birth. We are currently continuing the characterizations and plan to proceed on with the proposed carcinogenesis experiments.

Body/Results

One of the first goals of the study was to produce our transgenics on a pure background strain. This is imperative for the successful execution of informative carcinogenesis experiments. The original mice were on a highly mixed background, SSIN: C3H. There are several problems with this since mixed background animals can display variable inter-litter traits and phenotypes, and large numbers of genetically identical animals are needed to successfully conduct carcinogenesis experiments. Additionally, C3H animals are often infected with MMTV and have increased levels of stochastic mammary tumors, thus confounding the results from carcinogenesis studies. We, therefore, decided to produce congenic mice, i.e., to place the transgene on a pure background. This requires multiple backcrosses with animals of a stable, pure background strain. We chose 4 different backgrounds as shown in Table 1, and have successfully produced animals on a high percentage ($\geq 97\%$) background in three of these.

Table 1 Strains of mice used to produce transgenics on a pure background

Strain	virgin phenotype	pregnant phenotype	breeding/health problems	continued
SSIN	+++	+	++	yes
Balb/c	+++	+/-	--	yes
FVB	+++	++	+++	no
C57Bl	+++	++	++	yes

Characterizations of glands harvested from these three lines of animals have continued and data are presented in figure 1, in the appendix. In figure 1, panels A& B show whole mounts

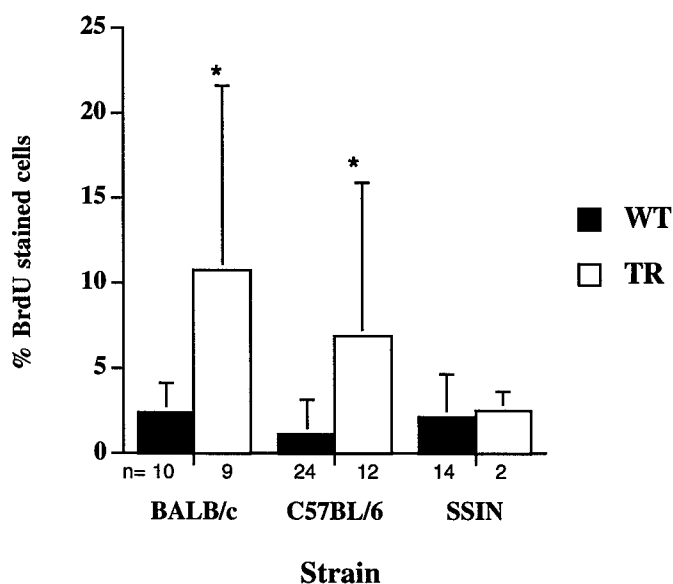
of abdominal mammary glands from sexually mature, virgin animals on a 97% Balb/c background. Figure 1 C& D are whole mounts of thoracic glands from animals on a 97% SSIN background, and E & F are abdominal glands from animals on a 98% C57Bl/6 background. Panels A, C and E show glands from wild type animals and B, D and F show tissue from transgenics. These data clearly demonstrate a profound level of reduction of mammary gland development in virgin, sexually mature transgenics as compared to age-matched, virgin wild type animals. Glands harvested from Balb/c transgenics had virtually no ductal arborization, and ductal development in SSIN and C57bl animals was also greatly reduced.

These effects can also be appreciated in histological sections as shown in figure 2, also in the appendix. In virgin glands from Balb/c animals, virtually no ductal structures can be appreciated, as compared to sections of wild type tissue (figure 2 A & B). Sections of glands from virgin SSIN and C57Bl also show reduced ductal structures in the transgenic tissues, but the differences are not as pronounced (data not shown).

Surprisingly, the level of disruption in glands from pregnant animals, harvested on the day of parturition (Day 0 of lactation), was very different. As shown in figure 2, development of the lactational phenotype was highly affected in glands from C57/bl (figure 2 E + F) and SSIN mice (figure 2 G + H), but not in Balb/c mice (figure 2 C + D). In fact, glands from pregnant Balb/c transgenics appear quite similar to those from wild type animals. Conversely, glands from transgenic SSIN and C57bl mice show appreciable reductions in differentiation compared to their wild type counterparts. These data suggest that maximal differences in susceptibility to mammary carcinogens would be expected in animals on a SSIN or C57bl background.

We have also evaluated proliferation and apoptosis in mammary tissues harvested from mice on the three different backgrounds. As shown in figure 3, proliferation, determined by

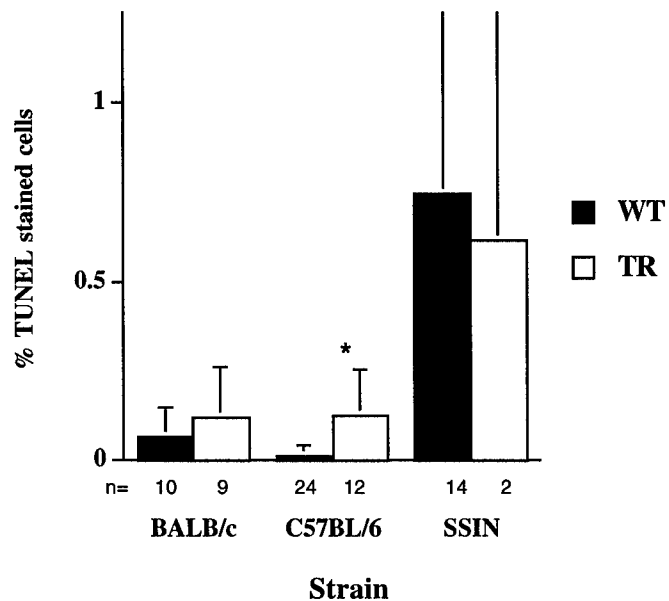
Figure 3 BrdU Incorporation in E2F-1 Virgin Glands



BrdU incorporation, was significantly reduced in wild type versus transgenic animals in Balb/c and C57bl mice (* = $p \leq 0.05$ from pooled t-test). The differences between wild types and transgenics of the SSIN strain did not reach statistical significance, however, probably due to the small number of animals available for analysis at the time of the preparation of this report. Although these animals are difficult to produce, we intend to improve these results by adding additional mice of this background strain.

Levels of apoptosis were determined by TUNEL staining and are shown in figure 4, below. As shown in this graph, only C57bl animals displayed a statistically significant difference in apoptosis in wild type versus transgenic mammary glands. While this trend is also

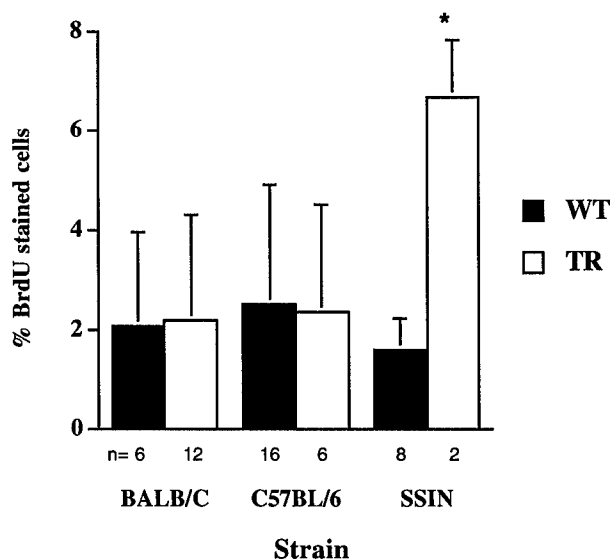
Figure 4 TUNEL Staining in E2F-1 Virgin Glands



evident in tissues from Balb/c mice, these results did not yet reach statistical significance. Again, the number of SSIN animals is too low at this time to generate meaningful data.

We also evaluated glands from pregnant animals, harvested on the day of parturition (lactation day 0). As shown in figure 5, proliferation in pregnant glands did not appear to be

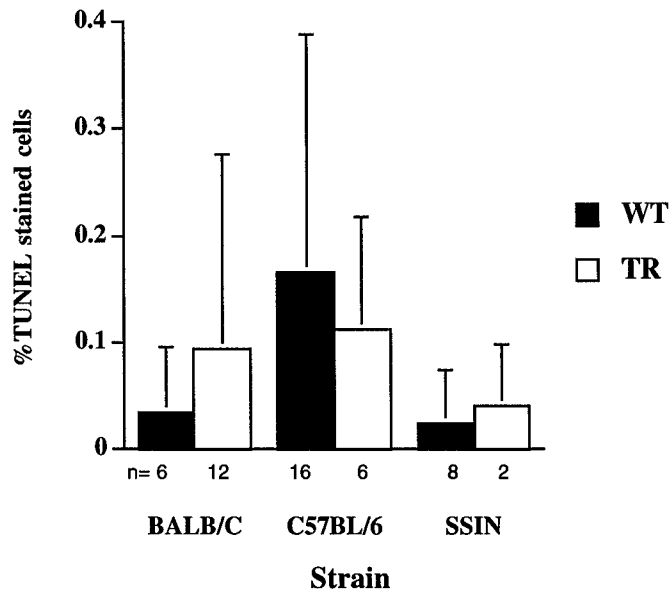
Figure 5 BrdU Incorporation in E2F-1 Pregnant Glands



significantly different in wild type versus transgenic glands. The exception again appeared to be in mice of the SSIN strain, but these numbers are small, as previously mentioned.

Apoptosis was also not significantly different in wild type and transgenic glands as

Figure 6 TUNEL Staining in E2F-1 Pregnant Glands



shown in figure 6. At present, there still appears to be an undesirable amount of animal to animal variation, perhaps accounting for the lack of significant differences. We will continue to produce animals on increasingly pure backgrounds in an effort to reduce this variability and provide statistically significant data.

Since we saw a profound effect of transgene expression on ductal arborization in virgin animals, we were concerned about the expression of the K5 promoter at very early stages of development. Little is actually known about expression of this and other myoepithelial antigens perinatally. We evaluated this by immunohistochemical staining for K5 and K8 antigens in tissues harvested from newborns and 2.5-day-old pups. As previously pointed out, K5 or Keratin 5, expression in the mammary gland is found in basal or myoepithelial cells, exclusively. Conversely, K8 is an epithelial antigen, expressed by adjacent glandular cells. Results from these studies are shown in figure 7, in the appendix. As shown in figure 7 panels A & B, rudimentary mammary gland buds are identifiable in the ventrum of a newborn pup. Importantly, separate myoepithelial and epithelial populations, in expected relative anatomic positions, are also appreciable in the newborns (panels C, D, E) and 2.5 day old pups (panels F, G and H). We used a double staining technique in which K5 was localized by an antibody linked to FITC, resulting in green fluorescence of myoepithelial cells. The K8 positive epithelial cells were localized with a Cy-5 linked antibody, identified by red fluorescence. If both antigens had been present in the same cells, the overlay of red and green fluorescence would have given a yellow image. However, since the two cell populations were distinct, separate green and red fluorescence can be appreciated. To our knowledge, this is novel data that demonstrates that K5 directed transgenes are affecting exclusively myoepithelial cells, and supports our hypothesis that myoepithelial cells contribute to mammary ductal development.

Key Research Accomplishments

- Production of lines of transgenic animals on “pure” background, necessary for carcinogenesis experiments.

- Characterization of phenotypes of different background strains to determine the most appropriate for future studies.
- Demonstration of profound effects of K5 directed transgene expression on glandular development in all strains of virgin transgenics, and in 2 of 3 strains of pregnant transgenics.
 - In virgin animals, ductal arborization is profoundly curtailed, and
 - Development of lactational differentiation is interrupted in animals on C57Bl and SSN backgrounds.
- Validation of our hypothesis that myoepithelial (K5 positive) cells are affecting glandular development, by identification of separate K5 and K8 populations in mammary tissue harvested from newborn and 2.5 day old pups.

Reportable outcomes

All research accomplishments described above are reportable, and the figures included in this report are being prepared for manuscript submission. In particular, the immunolocalization of separate K5 and K8 populations will be of interest to the scientific community, especially those studying mammary development.

To date we have presented data from these studies at two international meetings, one at the American Association for Cancer Research (1999) and one at The Endocrine Society (2000). Abstracts are attached.

As a result of this award we have been able to develop and characterize an important and unique model for the study of mammary myoepithelial function, and the resulting data will also be used to support a future R01-type application to the NIH.

Conclusions

At this time, we can conclude that alteration of myoepithelial cells through K5 directed overexpression of E2F-1 has a profound effect on ductal arborization and development of lactational phenotype in transgenic mice. There is also substantial variation in these effects between animals of different background strains. Importantly, the hypothesis that myoepithelial cells mediate these developmental effects was supported by K5 and K8 double staining of rudimentary mammary tissues from newborn and 2.5 day old pups. These double stains unequivocally demonstrate that separate populations of K5 and K8 expressing myoepithelial and epithelial cells, respectively, are present perinatally. Based on these results, the differences in susceptibility to carcinogenesis would be expected to be greatest in animals on C57bl and SSIN backgrounds, because these were the strains demonstrating the most profound inhibition of development due to expression of the transgene. These data directs future studies, by identifying the best strains to conduct carcinogenesis experiments.

Appendices

1. Abstract of presentation at 90th Annual meeting of the American Association for Cancer Research. Overexpression of E2F-1 and *c-myc* in mammary myoepithelial cells inhibits glandular differentiation and lactation. Fuchs-Young, R., Gamage, S., Ramirez, Y., Gimenez-Conti, I., Conti, C., Johnson, D.
2. Abstract of presentation at 82nd Annual Meeting of The Endocrine Society. Transgenic mouse models indicate that myoepithelial cells regulate mammary development and differentiation. R. Fuchs-Young, S. Gamage, D. Johnston, I. Gimenez-Conti, D. Johnson, C. Conti. UT MD Anderson Cancer Center, Science Park Research Division, Smithville, TX 78957
3. Figure 1 Whole mounts from sexually mature, virgin mice.

Panels A + B - abdominal glands from 97% Balb/c.

C + D - thoracic glands from 97% SSIN.

E + F - abdominal glands from 98% C57/Bl.

Panels A, C, E are wild type animals.

Panels B, D, F are transgenics.
4. Figure 2 Histological sections from virgin and pregnant (lactation day 0) mammary glands

Panels A + B H & E stained sections from virgin, sexually mature Balb/c mice.

C + D H & E stained sections of mammary glands from pregnant (lactation day 0) Balb/c mice.

E + F H & E stained sections of mammary glands from pregnant C57bl/6 mice.

G + H H & E stained sections of glands from SSIN mice.

Panels A, C, E & F are wild type animals.
Panels B, D, F & H are transgenics.

5. Figure 7

A + B H & E stained histological sections from newborn pups sacrificed within three hours of birth (Day 0). A, 10x, B, 40X.

C, D, E K5, K8 and combined K5 and K8 immunostaining of glands from newborn pups sacrificed on day 0, within three hours of birth.

F, G, H K5, K8 and combined K5 and K8 immunostaining of mammary glands from 2.5 day old pups.

6. Curriculum Vitae

Abstract of presentation at 90th Annual meeting of the American Association for Cancer Research, April 10-14, 1999, Philadelphia, PA.

Overexpression of E2F-1 and *c-myc* in mammary myoepithelial cells inhibits glandular differentiation and lactation. Fuchs-Young, R., Gamage, S., Ramirez, I., Gimenez-Conti, I., Conti, C., Johnson, D. UT/MD Anderson Cancer Center, Dept. of Carcinogenesis, Smithville, TX 78957.

Studies show that pregnancy at an early age is protective against breast cancer in humans and animals. The precise mechanisms underlying this protection are unclear, but increased structural and functional differentiation of the glands is involved. To study the role of myoepithelium in regulating differentiation and cancer susceptibility, we developed transgenic mice in which E2F-1 or *c-myc* overexpression is controlled by the K5 promoter and is directed to the myoepithelial cells of the mammary gland. Transgenic females from both lines were fertile but unable to nurse pups. Glands from post-pubertal, virgin E2F-1 transgenics had profoundly reduced ductal branching and alveolar bud formation compared to wild type littermates. Mammary glands from E2F-1 transgenics had significantly increased levels of apoptosis but reduced proliferation. Glands from pregnant E2F-1 transgenics were hypoplastic, had reduced alveolar development, increased interductal fat and lacked myoepithelial cells. These data suggest that overexpression of E2F-1 stimulated apoptosis and inhibited proliferation leading to loss of the myoepithelium. In contrast, mammary glands from *c-myc* transgenics had decreased levels of both apoptosis and proliferation. Virgin glands had normal amounts of ductal branching but no buds. Myoepithelial cells were present in glands from pregnant *c-myc* transgenics but alveolar morphology was disrupted and glandular epithelium attenuated. These results indicate that the myoepithelium plays a pivotal role in directing structural and functional differentiation of the mammary before and during pregnancy, and may, therefore, be an important mediator of susceptibility to carcinogenesis.

Abstract of presentation at 82nd Annual Meeting of The Endocrine Society, June 21-24, 2000, Toronto, Canada.

TRANSGENIC MOUSE MODELS INDICATE THAT MYOEPIHELIAL CELLS REGULATE MAMMARY DEVELOPMENT AND DIFFERENTIATION. R. Fuchs-Young, S. Gamage, D. Johnston, I. Gimenez-Conti, D. Johnson, C. Conti. UT MD Anderson Cancer Center, Science Park Research Division, Smithville, TX 78957

Studies indicate that pregnancy is protective against mammary cancer in rodents and humans, and that this effect is associated with structural and functional differentiation. To study the role of myoepithelium in achieving the protective effects of pregnancy, we have developed transgenic mice in which overexpression of E2F-1 or *c-myc* in the mammary gland occurs exclusively in myoepithelial cells. Although female transgenics are fertile, overexpression of E2F-1 or *c-myc* disrupted myoepithelial function, inhibited mammary differentiation and rendered mothers unable to nurse their pups. Glands from pregnant TR females were hypoplastic and underdeveloped compared to wild type (WT) littermates. In K5 E2F-1 transgenics, virgin glands had severely diminished ductal development and branching. Glands from pregnant E2F-1 TRs had greatly reduced alveolar development and increased interductal fat compared to WT. TUNEL analyses indicated that E2F-1 overexpression induced apoptosis of myoepithelial cells, which were virtually absent in glands harvested shortly after parturition, suggesting that these necessary cells were not readily replenished. In *c-myc* transgenics, glands from virgin animals were mildly affected, but glands from pregnant TR mice were highly disorganized with disrupted alveolar structure. In WT animals, an increase in proliferation and a decrease in apoptosis accompany development of the lactational phenotype, but the opposite was seen in tissues harvested from *c-myc* transgenics. In pregnant *c-myc* TR animals there was no significant increase in mammary BrdU incorporation but TUNEL-positive cells were significantly increased compared to virgins. Analyses of 4 congenic strains showed significant differences in the magnitude of transgene-induced alterations, although trends of decreased proliferation and increased apoptosis were similar. These results indicate that myoepithelial cells are important regulators of the development of the fully differentiated mammary phenotype of pregnancy, and may do so by mediating the balance of proliferation and apoptosis in glandular tissue. This effect is likely regulated by myoepithelial cells through control of extracellular matrix production and content. Since myoepithelial cells regulate the development of the protective lactational phenotype, this suggests that they are important mediators of the susceptibility of the gland to tumorigenesis and potential targets of therapeutic or protective strategies.

Figure 1
Fuchs-Young, Robin

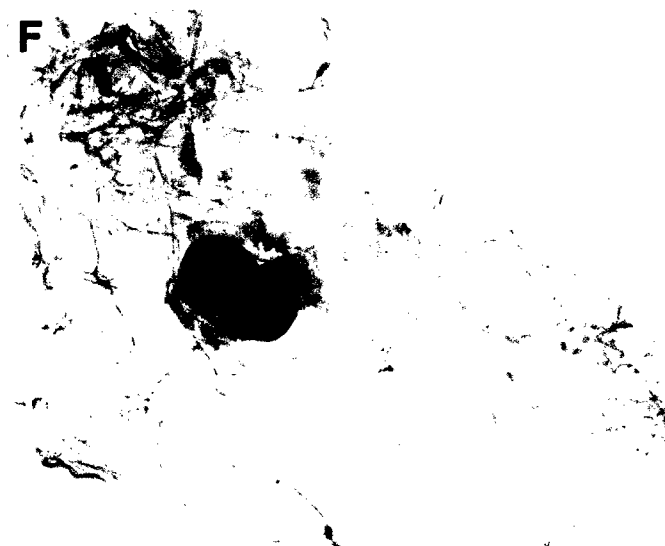
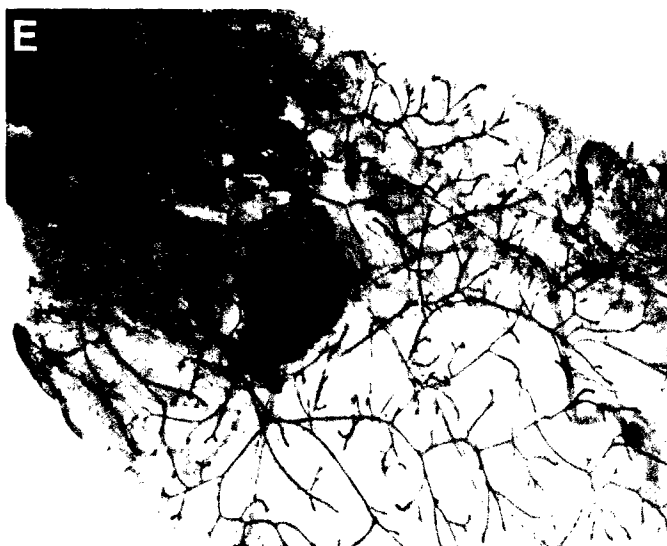
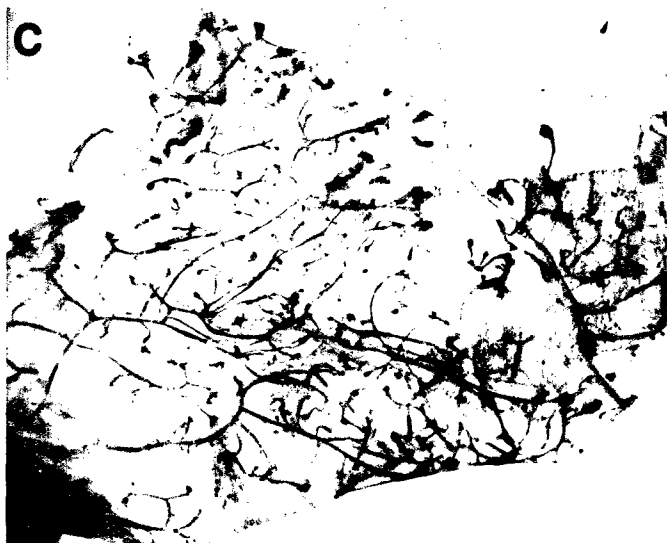


Figure 2
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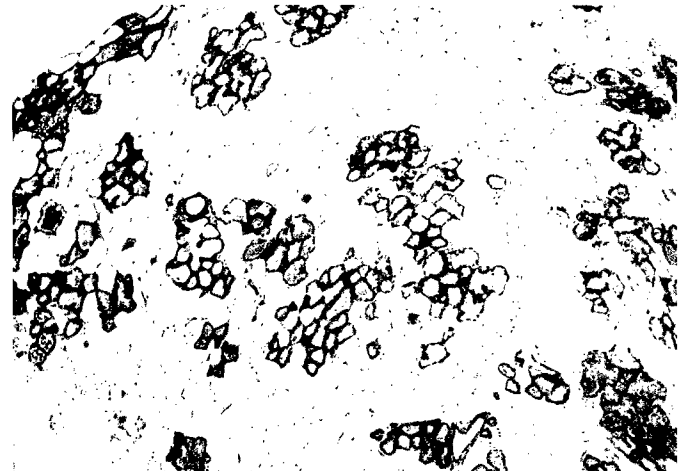
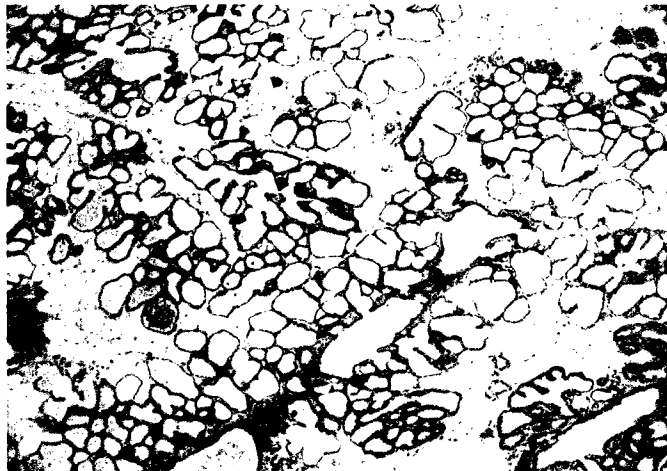
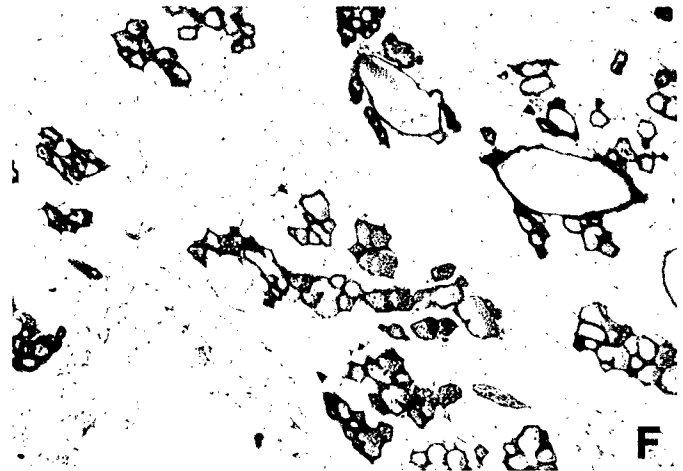
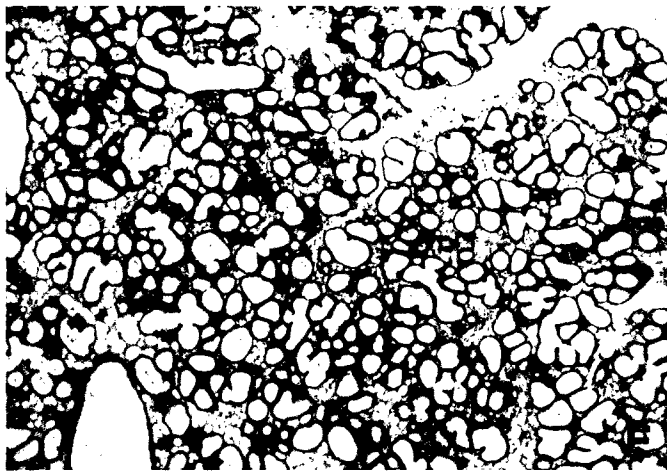
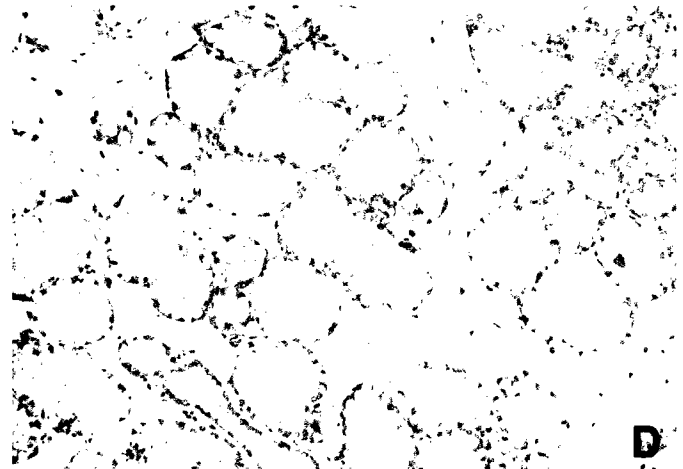
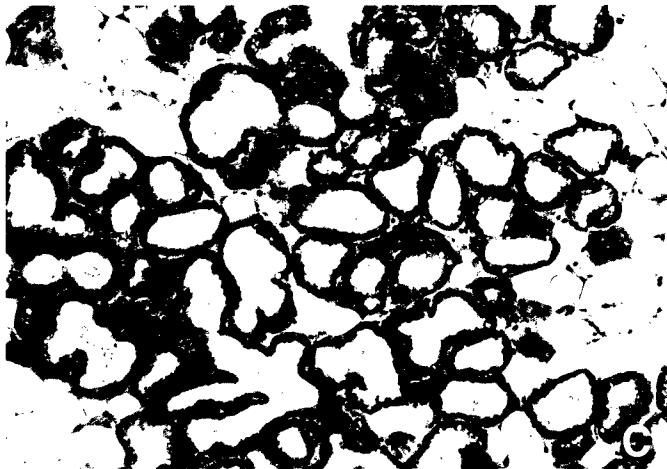
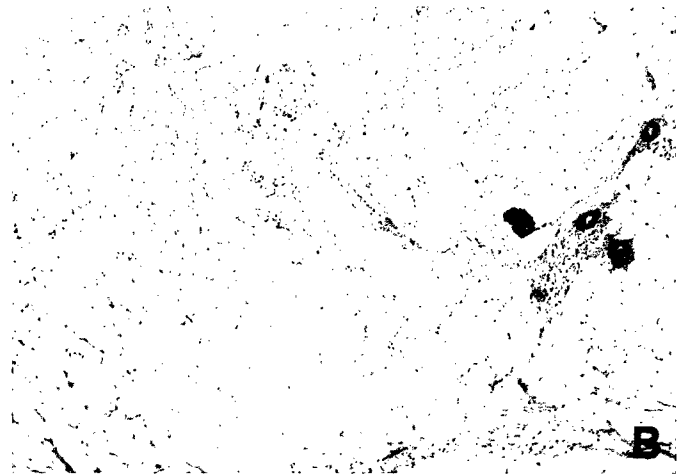
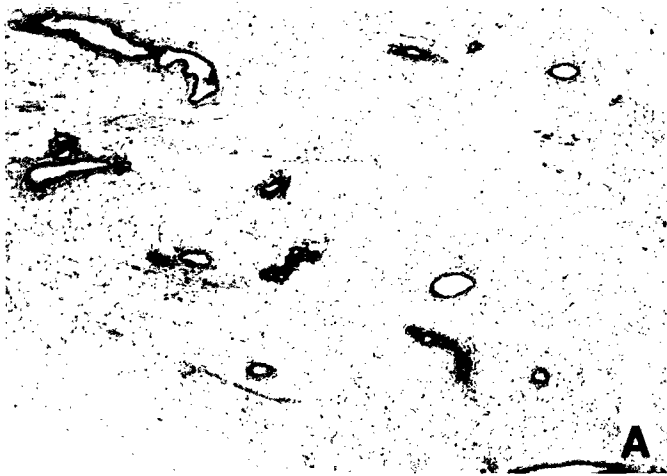
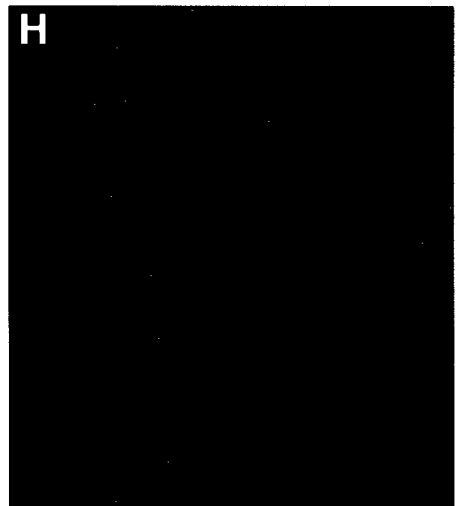
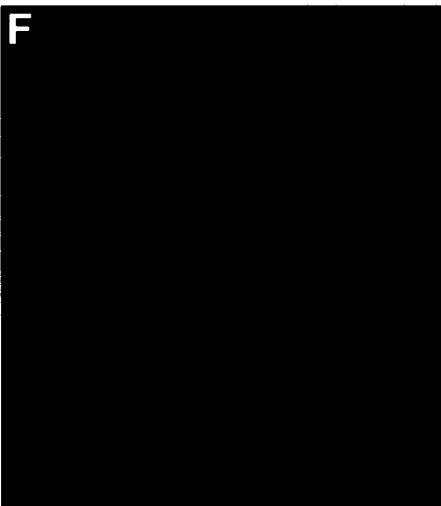
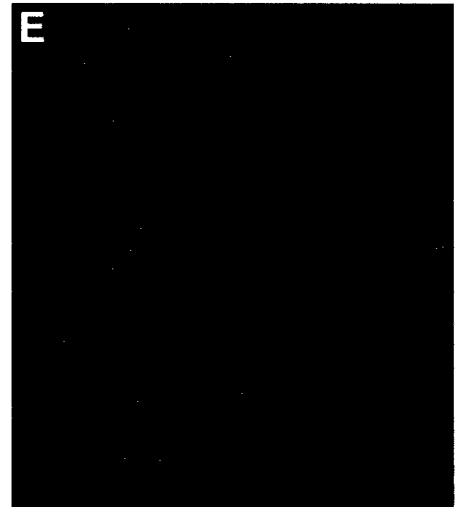
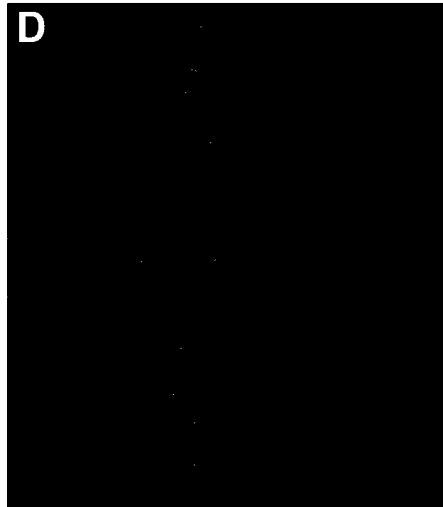
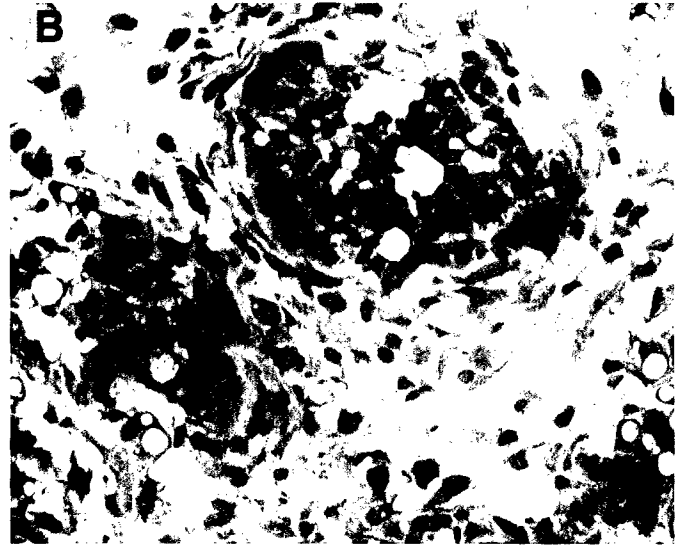
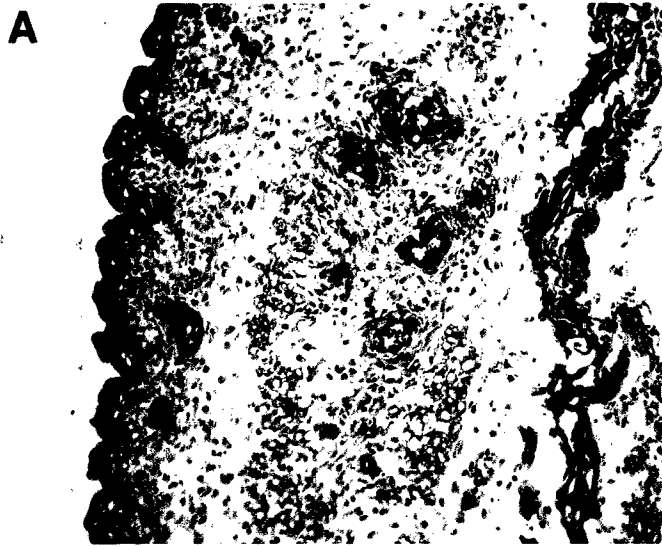


Figure 7
Fuchs-Young, Robin



Revised 9/25/01

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- 1996 Member, Ad Hoc, Endocrinology and Gene Therapy Study Section, California Breast Cancer Research Program
- 1996 Member, Ad Hoc, Molecular Genetic Review Panel (MGN-2), Department of Defense, Breast Cancer Research Program
- 1996-present Reviewer, Ad Hoc, for: Endocrinology, Cancer Research, Molecular Carcinogenesis
- 1997, 1998 Member, Ad Hoc, Basic Breast Biology Study Section, California Breast Cancer Research Program
- 1997, 1998, Member, Ad Hoc, Endocrinology Review Panel (END 1 or 2)
- 1999 Member, NIEHS Superfund Hazardous Substances Basic Research Program – SEP 4 review panel
- 1999, 2000,
2001 Department of Defense, Breast Cancer Research Program

Awards:

- National Institutes of Health, NCI, Postdoctoral Fellowship (NRSA)
- American Cancer Society Institutional Grant - predoctoral
- Vanderbilt University Graduate School Dissertation Research Award

Organization of Conferences:

- 1996 Organizer, Eli Lilly & Co., Opinion Leader Roundtable, Society for Gynecologic Investigation, Philadelphia, PA
- 1998 Session Chair - Keystone Symposium on Molecular and Cellular Biology, Nuclear Receptor Gene Family, Incline Village, NV

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Fuchs-Young, R., Everitt, J., Walker, C.L., and Davis, B. Discrimination of the tissue-specific biologic activity of therapeutic antiestrogens *in vivo*. In submission.

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Fuchs-Young, R., Hale, L., Layman, N., Short L., and Wilson, P. Comparison of the anti-proliferative effects of Raloxifene and other SERMs in the MCF-7 xenograft model.

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Fuchs-Young, R., Glasebrook, A.L., Short, L.L., Draper, M.W., Rippey, M.K., Cole, H.W., Magee, D.E., Termine, J.D., and Bryant H.U. (1995) Raloxifene is a Tissue-Selective Agonist/Antagonist that functions through the estrogen receptor. *Annals. NY Acad. Sci.*, 761:355-360.

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Fuchs-Young, R., Greene, G., and Mitchell, W.M. (1989) Site-directed antisera recognize functional domains of the estrogen receptor. The Tenth Annual Symposium on Reproductive Biology, Northwestern University.

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Fuchs-Young, R., Quinn, K., Rosner, M., and Greene, G. (1991) Autophosphorylation and downregulation of HER-2/neu in response to ligand and antibodies. Gordon Research Conference, Hormone Action.

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Sun, G., Colby, J., Lambertz, I., DeMayo, F., and Fuchs-Young, R. (2001) Role of progesterone receptor in estrogen-induced cell proliferation and gene expression in uterine myometrium. The Endocrine Society 83rd Annual Meeting.

Patents:

US Patent #5604248 Method for minimizing the uterotrophic effect of tamoxifen and tamoxifen analogs; issue: 2/18/97.

US Patent #5658931 Method for inhibiting mammalian breast carcinoma with tamoxifen and analogs thereof, and certain naphthyl compounds; issue: 8/19/97.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLLIS M. RINEHART
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